INTERLEUKIN-1 ANTAGONIST, DNA ENCODING SAME, AND ANTIBODIES THERETO

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part of
now U.S. Pat No.5,739,282
U.S. application No. 08/476,860, filed June 7, 1995, the

entire contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention is in the field of

biotechnology, and relates to a novel interleukin-1 (IL-1)

antagonist active against both IL-1α and IL-Iβ, to antibodies

specific to this novel IL-1 antagonist, to a new DNA sequence

encoding the IL-1 antagonist and to a method for obtaining a

IL-1 antagonist by recombinant DNA techniques. The present

invention also relates to the prophylactic, therapeutic and

diagnostic use of such a novel IL-1 antagonist in pathologies

associated with the IL-1 production.

BACKGROUND OF THE INVENTION

Two distinct genes, designated IL-1α and IL-1β,

encode for interleukin-1 (IL-1) proteins IL-1α and IL-1β,

respectively.

Interleukin IL-1α and IL-1β are pleiotropic

cytokines, and each, despite little sequence homology between

them, exerts a variety of similar effects on different tissues

and act on many human pathologies, and particularly on the

immune response of the organism and on inflammatory processes.

Both proteins have a molecular weight of about 17.5 kDa and

are synthesized as a larger precursor molecule having a molecular weight of about 31 kDa.

IL-1 proteins are potent inflammatory and pyrogenic cytokines that normally have beneficial effects, but that can also have extremely harmful effects on the organism. For example, IL-1 proteins participate in the pathogenesis of autoimmune pathologies, such as lupus erythematosus, and in particular, they are involved as mediators that provoke damage to tissues, as for example in rheumatoid arthritis.

Many of the biological effects of IL-1 are similar to those observed during sepsis. Recent studies demonstrated that the intravenous administration of IL-1 in a dosage range from 1 to 10 ng/kg gives rise to fever, sleepiness, anorexy, generalized myalgia, arthralgia and cephalea. Since IL-1 has pleiotropic biological activities, many of which negatively influence the organism, the powerful effects of IL-1 should be put under strict physiological control.

The synthesis of IL-proteins is inhibited by anti-inflammatory cytokines, prostaglandins and glucocorticoids and the existence of multiple levels of inhibition of IL-1 points to the necessity for strict control of this mediator. To date, IL-1 is the only cytokine for which an antagonist polypeptide for the receptor has been described; the third known component of the IL-1 family is the antagonist for the IL-1 receptor (IL-1ra).

All three components (IL-1α, IL-1β, IL-1ra) recognize and bind to the same receptor on the cell surface

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(IL-1R); the binding of IL-1α and IL-1β to IL-1R transmit a signal, whereas the binding of IL-1ra does not. There are two types of IL-1 receptors designated IL-1RI and IL-1RII. IL-1ra is a polypeptide which binds to IL-1RI, and also binds to IL-1RII with less affinity, without any agonistic activity.

IL-1ra production is induced in different types of cells, including mononuclear phagocytes, polymorphonuclear cells (PMN) and fibroblasts, by IgG, cytokines and bacterial products. Until now, only two molecular forms of IL-1ra have been identified and cloned:

1) secreted IL-1ra (sIL-1ra) contains a classical leader sequence of 25 amino acids giving a mature protein of 152 amino acids;

2) intracellular IL-1ra (icIL-1ra) lacks a leader sequence and it is predicted that this protein remains intracellular. sIL-1ra and icIL-1ra are generated from the same gene.

The human genes coding for IL-1α and IL-1 β and IL-1ra belongs to the same gene family and are clusterized on chromosome 2 mapping to the q12-q21 region (Eisenberg et al., Proc. Natl. Acad. Sci. USA 88:5232, 1991). The human IL-1ra gene has been partially sequenced and characterized. As shown in Fig. 5, the human IL-1ra gene contains in its 3′ region, three exons coding for the C-terminal common part of the three isoforms of IL-1ra (exons 2, 3 and 4 in Fig. 5) and in its 5′ region, three exons which can be differentially spliced to generate the three isoforms of the antagonist (exons ic1, ic2

and s1 in Fig. 5). The 3' part of the gene between the
promoter of the secreted isoform and the last exon of the
gene, as well the region upstream the first intracellular
specific exon, have been sequenced (bold line in Fig. 5)

5   (Butcher et al., J. Immunol.153:701, 1994; Lennard et al.,
Cytokine 4:83, 1992). On the other hand, the part of the gene
between the promoter region of sIL-ra and first exon of icIL-
1ra was unknown (thin line in Fig.5).

Transcripts of icIL-1ra1 originate from an

10   alternative starting site and from the splicing of a first
alternative exon into an internal splice acceptor site located
in the first exon of sIL-1ra. The predicted proteins are thus
identical except in their NH_2 ends, where the first 21 amino
acids of sIL-1ra are substituted by four amino acids in

15   icIL-1ra. The expression of transcripts encoding sIL-1ra and
icIL-1ra is regulated differently, and the biological
significance of icIL-1ra is still unclear.

Considering that IL-1 is involved in pathogenesis of
many diseases, it is evident that there is a need for

20   medicaments which are useful in limiting the harmful effects
of IL-1.

Citation of any document herein is not intended as
an admission that such document is pertinent prior art, or
considered material to the patentability of any claim of the

25   present application. Any statement as to content or a date of
any document is based on the information available to

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applicant at the time of filing and does not constitute an
admission as to the correctness of such a statement.

SUMMARY OF THE INVENTION

An object of the present invention is to provide an
IL-1 antagonist active against both IL-1α and IL-1β and
against a combination thereof.

Another object of the present invention is to
provide a DNA sequence encoding an IL-1 antagonist and a
method for obtaining such a novel antagonist by recombinant
DNA techniques.

A further object of the present invention is to
provide an antagonist in substantially purified form so as to
be suitable for use in pharmaceutical compositions active in
pathologies that can be treated by inhibiting IL-1.

Yet another object of the invention is to provide
antibodies specific for the IL-1 antagonist.

Further objects and advantages of the invention will
be evident in the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the DNA sequence and the amino acid
sequence, for the portion which is not in common, of the
icIL-1raII protein (SEQ ID N0:7 and SEQ ID NO:8) compared to
those of classic sIL-1ra (icIL-1raI; SEQ ID NO:6) and of
sIL-1ra (SEQ ID NO:4 and SEQ ID NO:5), and it further shows
the DNA sequence and the encoded protein sequence for the

portion of IL-1ra in common (SEQ ID NO:12 and SEQ ID NO:13).
Arrows indicate forward (IRA 1 and IRA 5) and backward (IRA 4)
oligonucleotides used for RT-PCR analysis, as described in the
text. The oligonucleotide IRA 5 recognizes only icIL-1reII
DNA. The asterisk indicates the stop codon.

Figures 2a-2c show the RT-PCR analysis of icIL-1raII
expression in different cell types. RNAs from 8387
fibroblasts (Fig. 2A), monocytes (Fig. 2B) and PMN (Fig. 2C)
were reverse-transcribed. Each DNA synthesis reaction was then
divided into two samples, one of which was amplified with
oligonucleotides IRA 5 (forward) and IRA 4 (backward) for
detection of icIL-1raII transcripts, and the other sample was
amplified with β-actin specific oligonucleotides (see Material
and Methods section). Amplified products were then examined
through an ethidium bromide-stained agarose gel. Amplified
products corresponding to β-actin are reported on the left
side of the standard and the amplified products corresponding
to icIL-1raII (on the right) are indicated by an arrow. The
specificity of these bands was confirmed by subcloning and
sequencing.

Figure 3 shows the Western blot analysis of
recombinant icIL-1raII. Cell lysates from COS cells
transfected with DNAs encoding icIL-1raI (lane 2) or
icIL-1raII (lane 3) or with an empty vector which does not
contain such DNA (lane 1) were examined by immunoblotting with
an anti-IL-1ra rabbit polyclonal antibody. Molecular weight
standards are indicated on the right.

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Figures 4A-4B show the effects of icIL-1raII on IL-1 induced expression of E-selectin in endothelial cells. Endothelial cells were treated with 0.1 (Fig. 4A) or 1 ng/ml (Fig. 4B) of human IL-1β, with or without 25-100 ng/ml of icIL-1raII or equivalent amounts of COS cell lysates obtained from cells which were mock transfected by means of an empty vector, as described in the Material and Method section. After 6 hours of incubation, the endothelial cells were examined for E-selectin expression by an ELISA test performed on adherent cells. The data reported are percentages of IL-1-induced E-selectin expression for the control.

Figure 5 shows the organization of the human IL-1ra gene. The boxes represent the exons (the size is not representative of the actual length in bp), where icI indicates the first exon of the intracellular form, icII indicates the new exon, s1 refers to the first exon of the soluble form, and 2, 3, and 4 indicate the 3' exons. The characterized isoforms described are schematically represented below the human IL-1ra gene and the lengths of the exons and introns are indicated above. The PCR primers that were used are indicated with arrows. The known sequence of the gene is indicated with a bold line and the previously unknown sequence of the gene is indicated with a thin line.

Figure 5 shows the organization of the human IL-1ra gene. The boxes represent the exons (the size is not representative of the actual length in bp), where icI indicates the first exon of the intracellular form, icII

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indicates the new exon, s1 refers to the first exon of the
soluble form, and 2, 3, and 4 indicate the 3′ exons. The
characterized isoforms described are schematically represented
below the human IL-1ra gene and the lengths of the exons and
5   introns are indicated above. The PCR primers that were used
are indicated with arrows. The known sequence if the gene is
indicated with a bold line and the previously unknown sequence
of the gene is indicated with a thin line.

Figure 6 shows the detection of icIL-1raII by
10   Western blot with rabbit serum. 100μg of COS cells are
transformed with icIL-1raII lysate (lane 1) or icIL-1raI (lane
2).

DETAILED DESCRIPTION OF THE INVENTION

The novel IL-1 antagonist was generated by inserting
15   a new 63 base pairs (bp) sequence between the first icIL-1ra
specific exon and the internal acceptor site of the first exon
of sIL-1ra and in frame with the DNA encoding icIL-1ra.

The present inventors found using RT-PCR that this
novel transcript is expressed in activated monocytes and
20   fibroblasts and in polymorphonuclear cells (PMN). Expression
in COS cells revealed that this novel antagonist is mostly
intracellular and has a molecular weight (MW) of approximately
25 kDa in SDS-PAGE.

The novel recombinant antagonist was demonstrated to
25   have IL-1 inhibitory activity.

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The known icIL-1ra will be referred to as icIL-1ra type I (icIL-1raI), and the novel antagonist, described here and the object of the present invention, will be referred to as icIL-1ra type II (icIL-1raII).

Examples of pathologies in which the novel antagonist according to the present invention can be advantageously used for prophylactic, therapeutic or diagnostic purposes are rheumatoid arthritis, septic shock, acute myelomonocytic leukemia, immunological reaction of transplant against host, acquired immunodeficiency syndrome (AIDS), ulcerative colitis and all autoimmune diseases in general.

An embodiment of the invention is the administration of a pharmacologically active amount of icIL-1raII to people having a high risk of developing pathologies that can be treated by inhibiting IL-1 or to people already showing pathologies like sepsis. An example of the category cited above are patients waiting for a surgical operation.

Any route of administration compatible with the active principle can be used, but parenteral administration is particularly preferred because it permits systemic effects in a short period of time. For this reason, it is preferable that the administration of an intravenous bolus is given just before, during or after the surgical operation. The dose of icIL-1raII to be administered depends on the age, weight and the individual response of the patient. The dosage can be

between 0.05 and 30 mg/Kg body weight and the preferable dose is between 0.1 and 10 mg/Kg body weight.

The pharmaceutical composition for parenteral use can be prepared in injectable form and comprises an active principle and a suitable vehicle. Vehicles for the parenteral administration are well known in the art and comprise, for example, water, saline solution, Ringer's solution and dextrose. The vehicle can contain smaller amounts of excipients in order to maintain stability in solution and isotonicity.

The preparation of the above-cited solutions can be carried out according to the ordinary modalities and preferably the icIL-lraII content will be between 1 mg/ml and 10 mg/ml.

Further examples of pathologies wherein the novel antagonist according to the invention can be advantageously used for prophylactic, therapeutic, or diagnostic purposes are rheumatoid arthritis, septic shock, acute myelomonocytic leukemia, immunological reaction of transplants against host, acquired immunodeficiency syndrome (AIDS), ulcerative colitis and all autoimmune diseases in general.

It should be understood that when the term "antibodies" is used with respect to the antibody embodiments of the present invention, this is intended to include intact antibodies, such as monoclonal or polyclonal antibodies, as well as proteolytic fragments thereof such as the Fab or F(ab')$_2$ fragments. Furthermore, the DNA encoding the variable

region of the antibody can be inserted into other antibodies to produce chimeric antibodies (see, for example, U.S. Patent 4,816,567) or into T-cell receptors to produce T-cells with the same broad specificity (see Eshhar, Z. _et al._, _Br. J. Cancer Suppl._, 10:27-9 (1990); Gross, G. _et al._, _Proc. Natl. Acad. Sci. USA_, 86:10024-8 (1989)). Single chain antibodies can also be produced and used. Single chain antibodies can also be produced and used. Single chain antibodies can be single chain composite polypeptides having antigen binding capabilities and comprising a pair of amino acid sequences homologous or analogous to the variable regions of an immunoglobulin light and heavy chain (linked V_H-V_L or single chain F_v). Both V_H and V_L may copy natural monoclonal antibody sequences or one or both of the chains may comprise a CDR-FR construct of the type described in U.S. Patent 5,091,513 (the entire contents of which are hereby incorporated herein by reference). The separate polypeptides analogous to the variable regions of the light and heavy chains are held together by a polypeptide linker. Methods of production of such single chain antibodies, particularly where the DNA encoding the polypeptide structures of the V_H and V_L chains are known, may be accomplished in accordance with the methods described, for example, in U.S. Patents 4,946,778, 5,091,513 and 5,096,815, the entire contents of each of which are hereby incorporated herein by reference.

A molecule which includes the antigen-binding portion of an antibody, is intended to include not only intact

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immunoglobulin molecules of any isotype and generated by any animal cell line or microorganism, but also the reactive fraction thereof including, but not limited to, the Fab fragment, the Fab' fragment, the $F(ab')_2$ fragment, the variable portion of the heavy and/or light chains thereof, and chimeric or single-chain antibodies incorporating such reactive fraction, as well as any other type of molecule or cell in which such antibody reactive fraction has been physically inserted, such as a chimeric T-cell receptor or a T-cell having such a receptor, or molecules developed to deliver therapeutic moieties by means of a portion of the molecule containing such a reactive fraction.

The antibodies and molecules which include the antigen-binding portion of an antibody specific for icIL-1raII according to the present invention can be used to purify icIL-1raII, such as by affinity chromatography. The availability of icIL-1raII specific antibodies also provides an invaluable tool in pathophysiology, where preliminary experiments in the laboratory of the present inventors suggest that icIL-1raII is indeed expressed under inflammatory conditions in humans.

Having now generally described the invention, the same will be more readily understood through reference to the following example which is provided by way of illustration and is not intended to be limiting of the present invention. In the section that follows, some methods for obtaining the invention will be described, although equivalent materials and methods can be used. The following examples are therefore

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purely illustrative and are non-limiting examples of the invention.

Example 1

Cloning and characterization of icIL-lraII

MATERIALS AND METHODS

Reagents

The following commercially available reagents were used for the culture and separation of cells: pyrogen-free saline and distilled water for clinical use; RPMI 1640 medium; DMEM medium; M199 medium; L-glutamine; Percoll; Ficoll-Hypaque; aseptically collected fetal calf serum; endothelial cell growth supplement (ECGS) prepared from bovine brain; Heparin. All reagents contained less than 0.125 EU/ml of endotoxin as confirmed by the Limulus amebocyte lysate assay.

Cells

Human circulating PMN and monocytes were separated from the peripheral blood of healthy donors by centrifugation on a discontinuous (46% for monocytes and 62% for PMN) gradient of isosmotic (285 mOsm) Percoll as described in Colotta F., Peri G., Villa SA., Mantovani A., Rapid killing of actinomycin D treated tumor cells by human mononuclear cells. *J. Immunol.* 132:936, 1984. Cells were recovered at the interface, washed twice in saline, and resuspended in medium.

PMN and monocytes recovery was higher than 90% and the purity was higher than 98% as assessed by morphological examination of stained cytocentrifuged cells. The cell culture

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medium routinely used for PMN and monocytes was RPMI 1640 with 2mM L-glutamine and 10% FCS.

Human endothelial cells (EC) were obtained from umbilical veins and cultured as described in detail in the literature (Allavena P., Paganin C., Martin-Padura I., Peri G., Gaboli M., Dejana E., Marchisio P.C., Mantovani A., Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium, *J. Exp. Med.*, 173:439, 1991).

Confluent cells at 2nd-5th passage maintained in M199 medium with 10% FCS supplemented with ECGS (50 μg/ml) and Heparin (100 μg/ml were routinely used.

COS cells were cultivated in DMEM medium with 10% FCS and 8387 fibroblast cells in RPMI 1640 medium with 10% FCS.

After the appropriate treatment, the cells were examined for IL-1ra mRNA or IL-1ra protein as described below.

RT-PCR

Total RNA was extracted by the guanadium isothiocyanate method with minor modifications.

RT-PCR was performed as described in Colotta F., Polentarutti, N., Sironi M., Mantovani A., *J.Biol.Chem.*, 267:18278, 1992.

Briefly, 1 μg RNA was reverse transcribed in reverse transcriptase buffer (5 mM MgCl$_2$, 50 mM KCl, 10 mM Tris-HCl; pH 8.3) with 2.5 mM random hexamers, 1 mM each deoxynucleotide

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triphosphate, 1 unit/ml RNase inhibitor, and 2.5 units/ml moloney murine leukemia virus transcriptase (Perkin Elmer Cetus, Norwalk, CT). Samples were incubated for 10 min at 25°C and then at 42°C for 45 min. Then, a specific pair of

5   primers, designed to amplify cDNAs encoding icIL-1raI or icIL1raII, were added to the cDNA reaction, and, as an internal control, human β-actin was added.

Amplification was carried out in 2 mM $MgCl_2$, 50 mM KCl, 0.2 M of each deoxynucleotide triphosphate, 2.5 units/100

10   ml Taq polymerase (Perkin Elmer Cetus) and 4 mg/ml of each specific primer (see below). Amplification (30 cycles) was carried out in an automated thermal cycler (Perkin Elmer Cetus) at 95°C, at 55°C, and at 72°C for 1.5 min each. Amplified products were run through a 1% ethidium bromide-

15   stained agarose gel along with molecular weight standards (Boehringer Mannheim, Mannheim, Germany).

Oligonucleotides were synthesized by the phosphoramidite method. The sequences of oligonucleotides used to selectively amplify icIL-1ra were identical to those

20   described in Haskill S. et al., *Proc. Natl. Acad. Sci.*, USA, 88:3681, 1991. In particular, oligonucleotides GM397 (indicated here as IRA 1) and GM368 (IRA 4) was used. For the icIL-1raII amplification, IRA 4 and IRA 5 (SEQ ID NO:1), which specifically recognizes the extra exon described here to be

25   included in the icIL-1raII sequence, were used.

For β-actin amplification the forward oligonucleotide is presented in SEQ ID NO:2, corresponding to

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nucleotides 60-79 of β-cDNA. The backward oligonucleotide
presented in SEQ ID NO:3, is complementary to nucleotides
430-449. The amplification products were subcloned (TA
Cloning System, Invitrogen, San Diego, CA) and sequenced by
5   the dideoxy chain termination method.

Analysis of genomic DNA

        Genomic DNA was extracted from human circulating
monocytes following standard protocols. 0.5 mg of genomic DNA
was amplified at 94°C for 1 min., 60°C for 1 min. and 70°C for
10   4 min. Oligonucleotide primers used were IRA1 (see above),
IRA9 (SEQ ID NO:14), IRA 7 (SEQ ID NO:15) and IRA10, which is
identical to B(BS1.1). Amplification products were subcloned
(TA Cloning System, Invitrogen, San Diego, CA) and sequenced
by the chain termination method. Analysis of the sequence was
15   performed with the Proexplore software package (Oxford
Molecular Ltd., Oxford, UK).

Generation of anti-icIL-1raII antiserum

        The peptide of SEQ ID NO:16 was conjugated to BSA by
coupling with glutaraldehyde. 15 mg of protein were injected
20   three times into rabbits s.c. at two week intervals. The
antiserum was tritiated by ELISA on peptide-coated plates.

Expression of icIL-1ra products in COS cells

        The cDNAs containing 32 bp of the 5'-untranslated
region, the complete open reading frame and 6 bp (including

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the stop codon) of the 3'-untranslated region of both the icIL-1ral and icIL-1raII were obtained by RT-PCR with oligonucleotides IRA 4 and IRA 5 as described above and then ligated back into the pSF5 expression vector. Fidelity of reverse transcription and amplification was verified by sequencing.

The plasmids containing the cDNA in the correct orientation were purified on CsCl gradient and then transfected into COS cells by the calcium precipitate method as described in Sambrook J. et al., Cold Spring Harbor Laboratory Press, 1989.

After two days, culture supernatants and sonicated cell lysates were examined by ELISA or immunoblotting as described below. An empty plasmid (not transfected) was used as a control.

Identification of immunoreactive IL-1ra

A commercial ELISA test (Amersham, Buckinamshire, UK) that identifies both sIL-1ra and icIL-1ra was used. For the Western blot analysis, polyclonal antisera of two rabbits and of one goat were used. COS cell lysate samples and supernatants were run on 12.5% SDS-PAGE electrophoresis and then blotted onto a nitrocellulose filter (Stratagene, La Jolla, CA, USA). Incubation with primary and secondary antibodies was carried out according to standard protocols. The primary antibody was an anti-IL-1ra rabbit polyclonal antibody. The secondary antibody was a goat anti-rabbit

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immunoglobulin fraction linked to horseradish peroxidase (Amersham). Immunoreactive protein fraction bands were revealed by a chemiluminescence-based procedure (ECL Detection, Amersham) according to manufacturer's instructions.

5   Il-1-induced expression of E-selectin on EC

Confluent EC cultivated in 96 well plates (Falcon) were incubated for 30 minutes with an amount of transfected COS cell lysate (see above) corresponding to 25 to 100 ng of recombinant IL-1ra (either icIL-1raI or icIL-1raII) as

10   assessed by a specific ELISA assay (Amersham).

As a control, an equal amount of COS lysate obtained from mock transfected cells was used in parallel. Next, EC were exposed for 6 hours to 0.1-1 ng/ml human recombinant IL-1β. The detection of E-selectin expression was made with

15   an ELISA assay on adherent EC with the anti-E-selectin monoclonal antibody BB1G-E2 as primary antibody and a rabbit anti-mouse Ig antiserum conjugated with horseradish peroxidase as a secondary antibody. Optical Density (O.D.) of the samples was determined by measuring the plates with a

20   spectrophotometer (Flow) at 405 nm wavelength.

RESULTS

Identification of icIL-1raII

Specific oligonucleotide primers were designed (indicated as IRA 1 and IRA 4 in Fig. 1) in order to obtain

25   the entire coding sequence of icIL-1ra (Fig. 1) by RT-PCR.

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Amplified products from human PMN were subcloned and
sequenced.

In addition to the previously known sequence of
icIL-1ra, the present inventors isolated a number of clones
whose sequences were identical to the published icIL-1ra
coding sequence, with the notable exception of an extra
sequence of 63 bp between nucleotides 132 and 133 of the
icIL-1ra sequence. Given the described exon-intron boundaries
of icIL-1ra, the extra sequence is inserted between the first
leader-less exon of icIL-1ra and the internal acceptor site of
the first exon of sIL-1ra (Fig.1).

The predicted amino acid sequence is shown in Fig.1.
The novel protein (herein referred to as icIL-1ra type II) has
the first three amino acids at the NH_2 terminus in common with
the classical icIL-1ra (icIL-1ra type I), followed by a new
sequence of 21 amino acids. The rest of the two proteins is
identical.

The entire sequence for each molecule is thus
generated by the junction of each specific portion with the
common sequence. For clarity, the DNA sequence of icIL-1ra
starts from nucleotide 91 of the published 5' untranslated
sequence, and only 6 bp of the 3' untranslated sequence are
reported. The common IL-1ra sequence (SEQ ID NO:9) starts
with the internal acceptor site located in the first exon of
sIL-1ra, corresponding to nucleotide 133 of the complete
icIL-1raI sequence and to nucleotide 88 of the complete
sIL-1ra sequence.

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With regard to questions related to the "Patentin"
program for the preparation of the sequences, a G nucleotide
was added in the first position of the sequence in order to
encode for the first amino acid Glu, and further in order to
5   avoid the formation of a stop codon in the inner side of the
sequence.

SEQ ID NO:10 reports the amino acid sequence of IL-
1ra for the portion which is in common and SEQ ID NO:11
reports the sequence of 21 amino acids representing an icIL-
10   1raII fragment not in common with the other IL-1ras.

Surprisingly, the junction with the internal
acceptor site of the first exon of sIL-1ra, both for sIL-1ra
and icIL-1raI and for icIL-1raII, always generated the same
amino acid residue, i.e. glutamic acid (Fig.1).

15   The most striking characteristic of the extra
inserted amino acid sequence is the presence of seven glycine
residues, six of which are consecutive. Glycine residues are
flanked on both sides by glutamic acid residues. icIL-1raII
consists of 180 amino acids, and the overall hydrophilic
20   pattern of icIL-1raII is similar to that of icIL-1raI, and
still lacks an hydrophobic leader peptide at the NH$_2$ terminus.

Expression of icIL-raII

To identify icIL-1raII transcripts, RT-PCR analysis
was performed with a pair of specifically designed
25   oligonucleotides (IRA 5 and IRA 4, Fig. 1), with an expected
amplified product of 533 bp. As shown in Fig.2A, transcripts

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encoding icIL-1raII were detectable in PMA-, IL-1- and
TNF-activated fibroblasts. A faint but detectable band was
evident in LPS-treated monocytes. PMN, either untreated or
activated (Fig.2C), also showed a very faint band of the
5   expected size. The specificity of amplified products
indicated in Figs. 2A-2C was confirmed by subcloning and
sequencing.

Expression of recombinant icIL-1raII

COS cells were transfected with the DNA sequence
10   encoding icIL-1raII and, by way of comparison, with that
encoding icIL-1raI. Next, cell lysates and supernatants were
examined by Western blot.

The polyclonal antisera used in these experiments
recognized icIL-1raII and icIL-1raI equally well (Fig.3).
15   Most, if not all, of iclL-1raII and icIL-1raI were found in
cell lysates. Recombinant icIL-1raI migrated as a predominant
band of 22 kDa, whereas icIL-1raII showed a mass of
approximately 25 kDa.

The recognition of icIL-1raII by anti-icIL-1raII
20   antiserum was checked by Western blotting icIL-1raII expressed
in COS cells (Fig. 6). As a comparison, soluble IL-1ra was
used with negative results.

Inhibition of IL-1B activity by recombinant icIL-1raII

Recombinant icIL-1raII was examined for IL-1
25   inhibiting activity using IL-1-induced expression of

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E-selectin on endothelial cells. This assay is sensitive
(detectable induction at 100 pg/ml IL-1, or less) and rapid
(6 hours incubation with IL-1).

5   Lysates of mock transfected COS cells did not
significantly reduce IL-1 activity. icIL-1raII had no
antagonistic activity. As shown in Figs. 4A-4B. recombinant
icIL-1raII inhibited in a dose-dependent fashion IL-1
activity. These data provide evidence that icIL-1raII is
indeed an inhibitor of IL-1.

10 Characterization of human IL-1ra genomic DNA

      PCR amplification of human genomic DNA was performed
in order to clone a portion of the human IL-1ra gene
containing the exon specific for icIL-1raII. The icIL-1ra
first exon had been previously localized approximately 9.4 kb
15 upstream of the sIL-1ra first exon (Butcher et al., J.
Immunol. 153:701 (1994)), but sequence information was
available only for a region of 6.2 kb located immediately
upstream of the sIL-1ra first exon (Butcher et al., J.
Immunol. 153:701 (1994); Lennard et al., Cytokine 4:83
20 (1992)). This intronic sequence did not contain the extra
exon specific for icIL-1raII. The laboratory of the present
inventors previously demonstrated that the extra exon specific
for icIL-1raII is localized in the remaining 3.2 kb genomic
sequence not yet available (Muzio et al., J. Exp. Med.,
25 182:623 (1995)). An IRA 10 oligonucleotide, which is colinear
with the most 5' sequence of the known intronic sequence

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(Butcher et al., J. Immunol. <u>153</u>:701 (1994), was designed and IRA 1 oligonucleotide, whose sequence is contained within the icIL-1ra first exon, were used in the PCR amplification.

A genomic fragment of 3.2 kb was amplified, cloned and sequenced (SEQ ID NO:17). The corresponding sequence of 63 nucleotides was found at a distance of 2037 bases downstream relative to the icIL-1raI first exon and was flanked by the exon-intron junction consensus AG and GT nucleotides (Mount, Nucl. Acid. Res. <u>10</u>:45 (1982)). The region immediately upstream relative to the new exon does not contain an ATG codon, thus suggesting that probably it is not a first exon, but rather an internal extra exon which can be spliced under precise stimuli (Fig. 5). Similarly to the known sequence of the IL-1ra gene, this region of the gene contains an Alu-type sequence between bases 760 and 1000.

An 86 bp sequence, tandem repeated, was present in the intron located downstream with respect to the first sIL-1ra specific exon (Lennard et al., Cytokine <u>4</u>:83 (1992)), where polymorphism in the number of repeats has been previously shown (Tarlow et al., Human Genet <u>91</u>:403-404 (1993)) and a specific allele has been associated with production of different cytokines (Danis et al., Clin Exp. Immunol. <u>99</u>:303-310 (1995)) and specific diseases (Clay et al., Hum. Genet. <u>94</u>:407-10 (1994)). Interestingly, the 86 bp repetition contains three potential binding sites, an α-interferon silencer A, a β-interferon silencer B and an acute phase response element. In the first intron, downstream with

respect to the icIL-1raI specific exon, a 13 bp sequence in position 1114 which is repeated in position 1142 was found where this sequence contains a LBP-1 consensus motif (Fig. 5 in the boxes).

DISCUSSION

The present inventors describe a novel molecular form of icIL-1ra which is generated by insertion of 63 bp between the first leader-less exon of icIL-1ra and the internal acceptor site of the first exon of sIL-1ra. Since the resulting protein is partially identical to classical icIL-1ra, with the exception of an extra sequence of 21 amino acids located in the NH_2 terminus of the molecule, the present inventors suggest that this novel form be designated as IL-1ra type II, and referring to the classical icIL-1ra sequence as icIL-1ra type I.

RT-PCR experiments demonstrated that icIL-1raII transcripts are inducible in monocytes and fibroblasts. Recombinant icIL-1raII expressed in COS cells had an apparent MW of approximately 25 kDa and an IL-1 inhibitory activity comparable to that exerted by icIL-1raI expressed under the same experimental conditions. Transcripts coding for icIL-1ra and sIL-1ra were generated from the same gene by means of differential splicing. icIL-1ra was generated from an alternative start of transcription of an exon inserted into an internal acceptor site of the first exon containing the leader sequence of sIL-1ra.

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The results obtained by the present inventors suggest a new organization for the IL-1ra gene, in which an extra exon is located between the first exon of, respectively, classical icIL-1ra and sIL-1ra. The presence of this extra exon generates a polypeptide molecule which lacks a signal peptide, but differs from icIL-1raI at its N-terminus by the insertion of 21 amino acids, and still remains inhibitory against IL-1.

The use of alternative splicing to generate different IL-1ra molecules appears to be highly regulated. icIL-1raII transcripts were induced by IL-1, TNF and phorbol esters in fibroblasts and by LPS in monocytes. In fibroblasts, phorbol esters were found to selectively induce icIL-1ra transcripts, whereas IL-1 and TNF induced both sIL-1ra and icIL-1ra mRNAs. In monocytes, IL-13, which augmented both transcripts of sIL-1ra and icIL-1raI, failed to induce icIL-1raII.

Finally, PMN, in which sIL-1ra and icIL-1ra are constitutively expressed and inducible, expressed very few transcripts, as demonstrated by RT-PCR. Overall, these results indicate that the mechanisms that induce the differential splicing, which generate the three forms of IL-1ra, are differentially regulated in response to external signals.

The amino acid sequence of the extra sequence described here is surprising in that it contains seven residues of glycine, six of which are consecutive.

Glycine-rich sequences are present in molecules with different biological activities, including the atrial natriuretic clearance receptor, the HOX11 homebox gene, the intermediate filaments keratins and nuclear proteins involved in centromere binding or RNA splicing.

Apart from glycine residues, however, no obvious homology was evident between these proteins and icIL-1raII in the amino acid sequence flanking the glycine-rich regions.

The IL-1 system shows an extraordinary level of complexity, and consists of two agonists, two receptors, one of which is an inhibitor of IL-1, and a receptor antagonist, for which at least three different molecular forms exist, as shown by the results obtained.

Although the biological significance of the intracellular forms of IL-1ra remains to be clearly established, the results reported here indicate that two different forms of icIL-1ra with different N-termini can be generated by alternative splicing in response to selected external stimuli.

The existence of multiple and complex levels of control of IL-1 points to the absolute requirement for a tight physiological control of the inflammatory potential of this cytokine.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the

spirit and scope of the invention and without undue
experimentation.

While this invention has been described in
connection with specific embodiments thereof, it will be
understood that it is capable of further modifications. This
application is intended to cover any variations, uses, or
adaptations of the inventions following, in general, the
principles of the invention and including such departures from
the present disclosure as come within known or customary
practice within the art to which the invention pertains and as
may be applied to the essential features hereinbefore set
forth as follows in the scope of the appended claims.

All references cited herein, including journal
articles or abstracts, published or unpublished U.S. or
foreign patents applications, issued U.S. or foreign patents,
or any other references, are entirely incorporated by
reference herein, including all data, tables, figures, and
text presented in the cited references. Additionally, the
entire contents of the references cited within the references
cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional
methods steps, known methods or conventional methods is not in
any way an admission that any aspect, description or
embodiment of the present invention is disclosed, taught or
suggested in the relevant art.

The foregoing description of the specific
embodiments will so fully reveal the general nature of the

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invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue

5   experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be

10   understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with

15   the knowledge of one of ordinary skill in the art.